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European and Mediterranean Plant Protection Organization
Organisation Européenne et Méditerranéenne pour la Protection des Plantes
PM 7/102 (1)

Diagnostics
Diagnostic

Curtobacterium flaccumfaciens pv. *flaccumfaciens*

Specific scope

This standard describes a diagnostic protocol for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.*[1]

Specific approval and amendment

Approved in 2011-09.

Introduction

Curtobacterium flaccumfaciens pv. *flaccumfaciens* is the causal agent of the bacterial wilt disease of *Phaseolus* spp. and is a systemic bacterium. The disease was first discovered in the United States (South Dakota) in the 1920s on *Phaseolus vulgaris* and subsequently recorded in Australia, Canada, Mexico, South America and Tunisia. It has a restricted distribution in Romania and Russia. Although it has been recorded in Belgium, Bulgaria, Greece, Hungary, Poland, Turkey and Ukraine, it has not established and is now considered absent from these countries. It has recently been reported in few fields in South-Eastern Spain (González *et al.*, 2005). Apart from this record, there are few recent records of *C. flaccumfaciens* pv. *flaccumfaciens* in the EPPO region.

No effective chemical methods are known against this disease; as *C. flaccumfaciens* pv. *flaccumfaciens* is a seed-borne bacterium, current control methods are based mainly on the use of healthy certified seeds. Economically, the most important host of *C. flaccumfaciens* pv. *flaccumfaciens* is *P. vulgaris* L., but the bacterium also attacks *P. coccineus* L., *P. lunatus* L. and *P. mungo* L.. Natural infections by *C. flaccumfaciens* pv. *flaccumfaciens* have been observed on *Vigna* spp. [*V. angularis* (Willd.) Ohiwi & Oashi, *V. radiata* (L.) R. Wilcz. and *V. unguiculata* (L.) Walsp. spp. *unguiculata*], on *Glycine max* (L.) Merr. and on *Pisum sativum* L. For more general information about *C. flaccumfaciens* pv. *flaccumfaciens*, see EPPO/CABI (1997).

This diagnostic procedure for *C. flaccumfaciens* pv. *flaccumfaciens* describes extraction from plant material or seeds, presumptive diagnosis with rapid tests and simultaneous isolation of bacterial colonies, identification of *C. flaccumfaciens* pv. *flaccumfaciens* putative isolates and, where relevant, determination of pathogenicity. A flow diagram describing the procedure is given in Fig. 1.

[1]Use of names of chemicals or equipment in these EPPO Standards implies no approval of them to the exclusion of others that may also be suitable.

Identity

Name: *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges) Collins & Jones.
Synonyms: ex-*Corynebacterium flaccumfaciens* subsp. *flaccumfaciens* (Hedges) Dowson.
Taxonomic position: Actinobacteria, Actinomycetales, Microbacteriaceae, Curtobacterium.
Notes on taxonomy and nomenclature: In addition to *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* and according to the most recent classification (Collins & Jones, 1984; Young *et al.*, 1996, 2004), the species *C. flaccumfaciens* includes the following pathovars: *betae* (Cfb), *oortii* (Cfo), *poinsettiae* (Cfp) and *ilicis* (Cfi). A pathovar *basellae*, affecting spinach, has been described (Chen *et al.*, 2000), as well as a pathovar *beticola* (Chen *et al.*, 2007). These new pathovars have been proposed, but not accepted so far by the ISPP Committee on the Taxonomy of Plant Pathogenic bacteria.
EPPO code: CORBFL.
Phytosanitary categorization: EPPO A2 list no.48, EU Annex II/B.

Detection

Disease symptoms

Young *Phaseolus* plants, 5–8 cm tall, may be attacked and are usually killed. If plants survive an early attack, or are infected at a later stage of growth, they may live throughout the season and bear mature seed. The disease is characterized by the wilting of leaves, or parts of them, initially during the warmest hours of the

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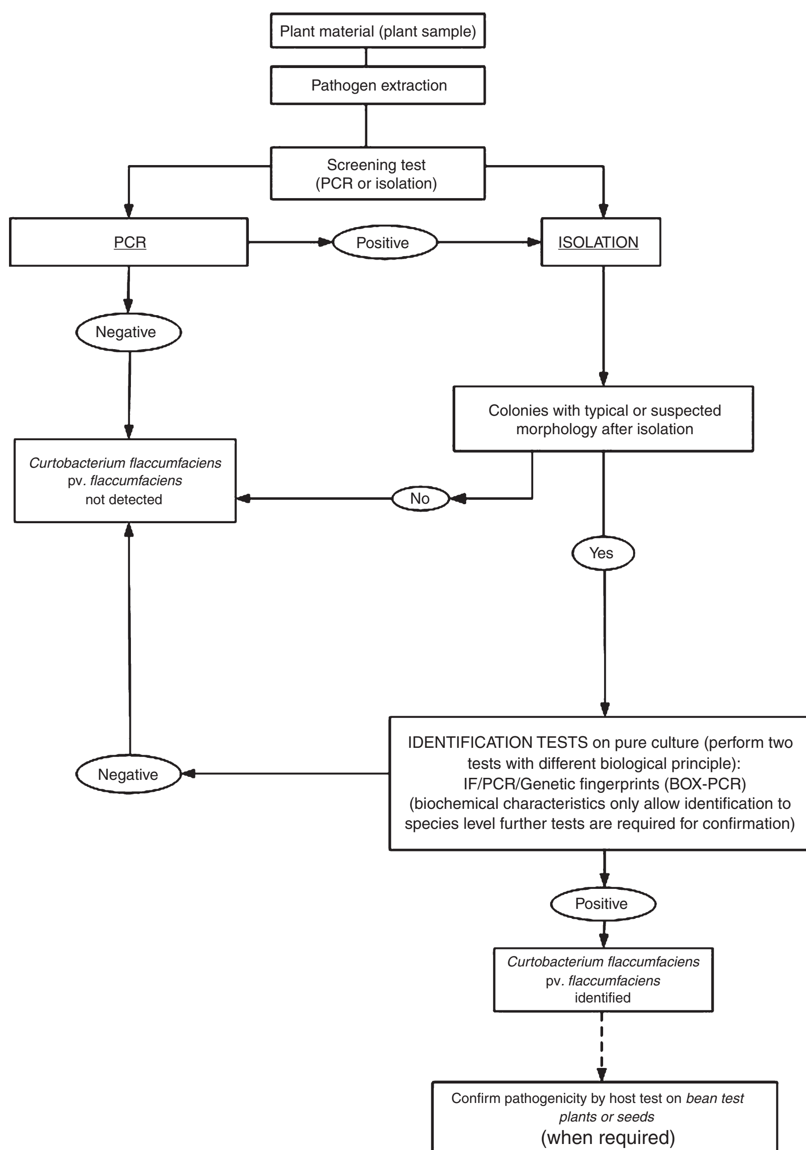

Fig. 1 Flow diagram for detection and identification of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens.*

day, followed by a recovery as the temperature drops in the evening. Wilting becomes permanent during the following days as a result of bacterial plugging of the vessels when the water supply is cut off; the leaves turn brown and then drop.

Occasionally, these typical wilting symptoms may be absent and replaced by golden-yellow necrotic leaf lesions closely resembling those of common blight, *Xanthomonas axonopodis* pv. *phaseoli* (Smith) (Young *et al.*, 1996); however, the lesion margin is more irregular in *C. flaccumfaciens* pv. *flaccumfaciens* infections. In general, there is no water-soaking of stems and leaves, as found in common blight and halo blight (*Pseudomonas syringae* pv. *phaseolicola*) (Burkholder) (Gardan *et al.*, 1992) infections.

On pods, the disease is much more conspicuous than in common blight. In fact, all the seeds in a pod may be infected, while the pod remains apparently healthy. This is due to the pathogen infecting the seed via the vascular system, following the sutures of the pods. The sutures may be discoloured with darkening, sometimes extending laterally. On young pods, water-soaked spots occasionally appear, the area turning either a yellowish-green or darker than the rest of the pod. On ripe pods, lesions are more evident, being an olive-green colour, in contrast to the yellow colour of the normal pod. It should be noted that seemingly vigorous plants may bear one or more shrivelled shoots, or infected pods which are hidden by healthy foliage.

Seeds of white-seeded cultivars, when infected systemically, are bright yellow, while the coloration is less intense in cultivars with coloured seed coats. There may be a small amount of yellow slime at the hilum, and seeds may be shrivelled. The colour mutants formerly described as *Corynebacterium flaccumfaciens*

subsp. *aurantiacum* and *violaceum* produce an orange and purple discoloration, respectively, in the seed coat.

Extraction

Extraction from symptomatic material

After surface sterilization [1 min in a sodium hypochlorite solution in water (0.5%, v/v)], small fragments of the stem (about 1 cm each), preferably taken from the darkened tissues, are placed in a mortar or a bag and covered with sterile physiological solution (SPS, 0.85% NaCl in distilled water) in approximately 300 µL for 1 cm of tissue; then the sample is roughly crushed with a pestle or comminuted with a sterile scalpel blade. About 10–20 min are needed for diffusion of the bacteria in the macerate, after which a loopful of the macerate is streaked on agar medium plates (see below for recommended media).

Symptomatic seeds are washed thoroughly under running water for 1 min and then washed in sterile distilled water. Symptomatic seeds (a few up to a dozen) are then placed in a Stomacher bag, covered with SPS (approximately 2 mL/symptomatic seed), to soak at approximately 4°C overnight. Seeds are then roughly crushed with the Stomacher or with a hammer, filtered to eliminate any seed particle (using a sterile gauze or Miracloth paper), then concentrated by centrifugation (6000 *g* at approximately 4°C for 20 min). The supernatant is discharged and the pellet resuspended in 1 mL SPS to obtain the final concentrate (FC). The FC is used for direct isolation or PCR tests.

Extraction from asymptomatic seeds

- Sample preparation

In principle, the total number of seeds to be tested and the maximum number of seeds to be processed per subsample depends on the maximum acceptable percentage of infested seed to be detected and the detection limit of the tests. As this information is not available for *C. flaccumfaciens* pv. *flaccumfaciens*, a number of samples statistically representative for the lot to be checked is collected. A sample of 1 kg, subdivided into five subsamples of 200 g each, is analysed twice (Maringoni *et al.* (2006).

Two methods are described below for seed extraction.

- If testing is performed to detect *C. flaccumfaciens* pv. *flaccumfaciens*, it is preferable to use only a method that 'crushes' the beans, as *C. flaccumfaciens* pv. *flaccumfaciens* is a systemic endophyte. If it is required, surface sterilization of bean seeds can be done by soaking in 70% ethanol for 15 s. As crushing produces more debris than soaking, the number of seeds per subsample may need to be reduced by about half for the same buffer volume.
- If testing is performed simultaneously for different pathogens, such as *P. savastanoi* pv. *phaseolicola*, *P. syringae* pv. *syringae* and *X. axonopodis* pv. *phaseoli* (epiphytic and subtegumental bacteria), the soaking method is more appropriate. No surface sterilization should be performed.
- Crushing method

Pathogen extraction is done by soaking each sample (5×200 g subsamples) in an appropriate volume of SPS. Bean seeds are placed in a sterile glass jar and covered with SPS (approximately 250–300 mL for a 200 g subsample), then kept overnight at approximately 4°C under gentle shaking. Soaking is followed by roughly crushing the beans with blades (Ultra-Turrax or similar homogenizer), set at a low speed for 3–5 min, in order to comminute the seeds. The crushed seeds are then filtered through a sterile gauze and the filtered fluid is centrifuged for 20 min at 10 000 *g* and approximately 4°C. The supernatant is discharged and the pellet is resuspended in 1.5–2 mL SPS to obtain the FC. The FC is used for direct isolation or PCR tests.

- Soaking method

Pathogen extraction is done by soaking the samples in an appropriate volume of SPS (1000–5000 seeds per litre, according to seed size). Bean seeds are placed in a sterile glass jar and covered with SPS, then kept overnight at approximately 4°C under shaking. The soaking fluid is centrifuged for 20 min at 10 000 *g* and approximately 4°C. The supernatant is discharged and the pellet is resuspended in 1.5–2 mL SPS to obtain the FC. The FC is used for direct isolation or PCR tests.

Screening tests

Direct isolation and PCR can be used as screening tests. An immunofluorescence protocol has been developed to be applied for seed tests (Calzolari *et al.*, 1987; Diatloff *et al.*, 1993). However both mono- and polyclonal antibodies raised and screened against *C. flaccumfaciens* pv. *flaccumfaciens* have been shown to be defective in specificity and sensitivity, failing to react with all the strains of *C. flaccumfaciens* pv. *flaccumfaciens* tested (Calzolari *et al.*, 1987; McDonald & Wong, 2000). A phytosanitary procedure, based on the indirect isolation of *C. flaccumfaciens* pv. *flaccumfaciens* by inoculation of bean seedlings with the FC, used to be recommended (EPPO, 1994), but it is time-consuming and has a quite low sensitivity (1.23×10^6 cells). Consequently these tests alone are not recommended for detection.

Direct isolation on agar media

To perform direct isolation, a minimum of two agar media are suggested: one general and one semi-selective. Aliquots of the FC and several tenfold dilutions are spread (100 µL per plate). Suitable media are yeast peptone glucose agar (YPGA); nutrient broth yeast (NBY) extract agar medium (Vidaver, 1967); and the semi-selective medium (SSM) developed by Tegli *et al.* (1998) on the basis of that of Mizuno & Kawai (1993). NBY and YPGA plates are incubated in the dark at 23°C for 2–3 days, while SSM plates are incubated at 30°C for 7–10 days. The composition of media is given in Appendix 1. The combination of NBY and SSM agar media usually gives good results.

After 2–3 days in culture on YPGA and NBY media, colonies of *C. flaccumfaciens* pv. *flaccumfaciens* are circular, 2–4 mm in diameter, smooth and with entire margins, more often convex and translucent, but sometimes also flat and semi-opaque. Their pigmentation is variable, depending on temperature and pH, and creamy yellow to bright yellow or orange colonies can be found. Thus, simple observation of visual features on agar media alone may be inconclusive, and several colonies should be selected for a more robust detection. A KOH test may be eventually

performed to choose Gram-positive colonies for further testing (Halebian *et al.*, 1981).

After 7–10 days of incubation on SSM, *C. flaccumfaciens* pv. *flaccumfaciens* colonies are 1–2 mm in diameter and are clearly detectable from the yellow colour in contrast with the violet background of the medium. A yellow halo surrounding the colony is sometimes present.

Other bean seed-transmitted pathogens, such as *P. savastanoi* pv. *phaseolicola* and *X. axonopodis* pv. *phaseoli*, are unable to grow on SSM medium.

Please note that the MSCFF medium is not recommended in this protocol, as validation experiments conducted in the Dutch National Reference Centre showed this medium to have a very low specificity.

PCR

Two PCR tests for the detection of *C. flaccumfaciens* pv. *flaccumfaciens* in bean seeds (Tegli *et al.*, 2002 and Guimaraes *et al.*, 2001) are described in Appendices 2 and 3, respectively. In case of positive PCR results on these samples, direct isolation of viable *C. flaccumfaciens* pv. *flaccumfaciens* cells on agar media should be performed.

Identification

Morphological characterization

Curtobacterium flaccumfaciens pv. *flaccumfaciens* is motile, with one to three lateral or polar flagella, non-spore-forming, occurring singly and in pairs, 0.6–3.0 × 0.3–0.5 μm in size. Characterization on the basis of biochemical features for *C. flaccumfaciens* pv. *flaccumfaciens* is up to species level. Biolog® was also used successfully (Maringoni *et al.*, 2006) to identify *C. flaccumfaciens* pv. *flaccumfaciens* via profiling of single-carbon-sources utilization, but no technical details are available.

Biochemical characteristics to confirm species identity: *Curtobacterium flaccumfaciens* are shown in Table 1.

Table 1 Biochemical characteristics for *Curtobacterium flaccumfaciens*

Test	Result
Gram stain	+
O/F metabolism of glucose	O: +; (F: −)
Catalase	+
Cytochrome *c* oxidase	−
Urease	−
Indole	−
Gelatin hydrolysis	+
Casein hydrolysis	+
Acid from mannose	+
Acid from maltose	+
Acid from inulin	−
Acid from erythritol	−
Acid from mannitol	−
Acid from inositol	+

−, negative result; +, positive result. Based on Komagata & Suzuki (1986) and Bradbury (1986).

Identification at pathovar level requires additional tests. API® 20 NE tests have been evaluated with a collection of strains of this pathogen, showing quite homogeneous results, although variability can be observed in one or more tests, and they can be used for its identification as shown in Table 2. The tests should be performed following the manufacturer's instructions and the results should be read after 48 h incubation at 25°C.

Serological methods

Immunofluorescence (IF) can be used to confirm the identity of putative *C. flaccumfaciens* pv. *flaccumfaciens* pure cultures. Commercial antisera are available (e.g. Florilab, Neogen-Adgen, Plant Research International). The IF test using bacterial suspensions of about 10^6 cells/mL is described in PM 7/97. Since there might be *C. flaccumfaciens* pv. *flaccumfaciens* isolates which are different from the main population and may react as negative to IF a confirmation of their identity may be done coupling IF with rep-PCR, using the BOX primer (Guimaraes *et al.*, 2003).

Fatty acid profiling

Although fatty acid profiling has been suggested to identify and characterize *C. flaccumfaciens* pv. *flaccumfaciens* (Stead *et al.*, 1992; Weller *et al.*, 2000; Dickstein *et al.*, 2001), it is not recommended for the following reasons. Key fatty acids include 15:0 iso, 15:0 anteiso, 16:0 iso, 16:0, 17:0 anteiso and 18:1 ω7*cis*. The first 5 of these are typical of a coryneform bacterium; 18:1 ω7*cis* is unusual in such bacteria, although commonly found in Gram-negative bacteria. 18:1 ω7*cis* is found in most strains of *C. flac-*

Table 2 Reaction of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in API® 20 NE

Test	Reaction
NO_3	−
TRP	−
GLU	−
ADH	−
URE	v
ESC	+
GEL	v
PNG	+
GLU	+
ARA	+
MNE	+
MAN	+weak
NAG	v
MAL	+
GNT	+
CAP	−
ADI	−
MLT	−/weak
CIT	−/weak
PAC	−

v, varible.

cumfaciens pv. *flaccumfaciens*. It is not found in any *Clavibacter* species. It is also found in some other *C. flaccumfaciens* pathovars and in *Curtobacterium* spp. However, non-pathogenic *Curtobacterium* spp. are common on many plant surfaces, and these bacteria often have close matches with *C. flaccumfaciens* pv. *flaccumfaciens* fatty acid profiles, limiting the usefulness of the test. If fatty acid profiling is used, further PCR-based analysis needs to be performed (see below for BOX-PCR and *C. flaccumfaciens* pv. *flaccumfaciens*-specific PCR protocols).

Molecular methods

PCR

The PCR protocols developed by Tegli *et al.* (2002) and Guimaraes *et al.* (2001) are described in Appendices 2 and 3, respectively.

Genetic fingerprinting

BOX-PCR is described in EPPO Standard PM 7/100 *Rep-PCR tests for identification of bacteria*.

Pathogenicity test

When required, confirmation and verification of the pathogenicity of identified *C. flaccumfaciens* pv. *flaccumfaciens* isolates is performed on bean plantlets or been seeds. These tests are described in Appendix 4.

Reporting and documentation

Guidance on reporting and documentation is given in EPPO Standard PM7/77 (1) *Documentation and reporting on a diagnosis.*

Further information

Further information on this organism can be obtained from:

A Calzolari, Servizio Fitosanitario Regionale dell'Emilia Romagna, Via di Corticella 133, 40129 Bologna (IT);

M Fuhlbohm, Farming Systems Institute, Queensland Department of Primary Industries, J. Bjelke Petersen Research Station, PO Box 23, Kingaroy (AU).

Acknowledgements

This protocol was originally drafted by S. Tegli, Dipartimento di Biotecnologie Agrarie (DiBA), Sezione di Patologia Vegetale, Laboratorio di Patologia Vegetale Molecolare, Via della Lastruccia 10, Polo Scientifico dell'Università degli Studi di Firenze, 50019 Sesto Fiorentino, Firenze, IT, email: stefania.tegli@unifi.it.

Feedback on this Diagnostic Protocol

If you have any feedback concerning this Diagnostic Protocol, or any of the tests included, or if you can provide additional validation data for tests included in this protocol that you wish to share please contact diagnostics@eppo.fr.

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Appendix 1 – Media

Yeast peptone glucose agar (YPGA) (Lelliot & Stead, 1987)

Yeast extract	5.0 g
Bactopeptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Distilled water	1.0 L

Dissolve ingredients and sterilize by autoclaving at 121°C for 15 min.

Nutrient broth yeast extract (NBY) agar (Vidaver, 1967)

Nutrient broth	8.0 g
Yeast extract	2.0 g
K_2HPO_4	2.0 g
KH_2PO_4	0.5 g
Glucose	2.5 g
Agar	15.0 g
Distilled water	1.0 L

Dissolve ingredients and sterilize by autoclaving at 115°C for 20 min.

Semi-selective medium (SSM) developed by Tegli *et al.* (1998) on the basis of that of Mizuno & Kawai (1993).

Rhamnose	5.0 g
Yeast extract	2.0 g
KH_2PO_4	0.5 g
K_2HPO_4	2.0 g
NH_4Cl	1.0 g
LiCl	10.0 g
$MgSO_4.7H_2O$*	0.25 g
Tris-HCl	1.2 g
Sodium azide*	2.0 g
Cycloheximide* (Sigma)	0.1 g
Polymixin B*	0.04 g
Bromocresolpurple (15% solution in ethanol)	1 mL
Agar	15 g
Distilled water	1.0 L

Adjust pH to 6.8 then autoclave for 15 min at 121°C.
*These components have to be filter-sterilized separately, without autoclaving, and then aseptically added to the basal medium at approximately 50–55°C, before pouring it into the plates.

Appendix 2 – CR test Tegli *et al.* (2002)

1. General information

1.1 Protocol based on Tegli *et al.* (2002) PCR technique for the specific detection of *C. flaccumfaciens* pv. *flaccumfaciens*.
1.2 Nucleic acid is prepared from the FC (following soaking) or from suspensions from pure cultures.
1.3 Specific primers for the amplification of *C. flaccumfaciens* pv. *flaccumfaciens*. CffFOR2-CffREV4 (EMBL Accession Numbers AJ318036 and AJ318037, respectively) (CffFOR2 5′-GTT ATG ACT GAA CTT CAC TCC-3′) (CffREV4 5′-GAT GTT CCC GGT GTT CAG-3′).
1.4 Amplicon size: 306 bp.
1.5 Enzyme used: 1U Taq DNA polymerase (Polytaq from Polymed s.r.l., Florence, IT).
1.6 The reaction mixture contains 156 μM of each dNTP.
1.7 Automated thermal cycler used (Delphy 1000™, Oracle Biosystems™, MJ Research Inc., Watertown, MA, US).

2. Methods

2.1 DNA extraction
2.1.1 DNA is extracted from the FC as plant inhibitors and fungicides/preservatives used to coat seeds might interfere with the PCR reaction. The test can also be used to identify pure cultures. There is no need to perform a traditional DNA extraction on pure cultures, a thermal lysis procedure is adequate. A single colony is resuspended in sterile bi-distilled water (100 μL per colony), heated at 95°C for 10 min, and then immediately cooled on ice for at least 5 min. As DNA template, 2 μL of the thermal lysed DNA are used for each reaction (25 μL final volume).

2.1.2 DNA extraction is performed using a Gentra Puregene Cell Kit (Qiagen) or Instagene Matrix (Biorad, Hercules, CA, USA), according to manufacturers' instructions.
2.1.3 The DNA extracted using a Puregene DNA extraction kit is resuspended in 50 µL TE buffer (10 mM Tris–HCl buffer, pH 8.0, 1 mM EDTA) (Sambrook *et al.*, 1989).
2.1.4 Samples obtained by both DNA extraction methods should be processed immediately; if not, they should be stored at approximately −18°C prior to PCR reactions.

2.2 PCR
2.2.1 Total reaction volume of 25 µL
10 ng of DNA or 2.5 µL DNA from thermal lysis,
20 mM Tris–HCl (pH 8.0),
50 mM KCl,
1.5 mM $MgCl_2$,
156 µM of each dNTP,
0.5 µM of each primer of the pair,
1U Taq DNA polymerase.
2.2.2 PCR cycling parameters:
An initial denaturation at 94°C for 3 min, after which 30 cycles of denaturation (1 min at 94°C), primer annealing (45 s at 62°C) and primer extension (30 s at 72°C) were performed, followed by a final extension at 72°C for 5 min.
Amplification products (2.5 µL each) should be separated by agarose gel electrophoresis, stained with ethidium bromide [1.4% (w/v) agarose gels with 1× TBE buffer] and visualized under UV light. A specific fragment of 306 bp should be amplified.

3. Essential Procedural Information

3.1 Controls:
For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively
- Negative isolation control (NIC) sterile distilled water and DNA extracted by washings of healthy bean seeds.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR-grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results
A sample will be considered positive if it produces an amplicon of 306 bp, provided that the contamination controls (NIC, NAC) are negative.
A sample will be considered negative if it produces no band, or a band of a different size (not 306 bp), provided that the PIC and PAC are positive.
Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data:
The sensitivity threshold is about 5 pg DNA.
4.2 Analytical specificity data
This test is specific for *C. flaccumfaciens* pv. *flaccumfaciens*. It has been developed using 17 strains of *C. flaccumfaciens* pv. *flaccumfaciens* isolated from *P. vulgaris* plants and seeds in USA, Germany, Hungary and Romania, and was shown not to cross-react with *Rhodoccocus fascians* pv. *phaseolicola*, *X. axonopodis* pv. *phaseoli*, *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oortii*, *C. flaccumfaciens* pv. *poinsettiae*, *Clavibacter michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *michiganensis* (two isolates), *C. michiganensis* subsp. *sepedonicus*, *Pseudomonas savastanoi* pv. *phaseolicola.*
4.3 Data on Repeatability
Not available
4.4 Data on Reproducibility
Not available

Appendix 3 – PCR test Guimaraes *et al.* (2001)

1. General Information

1.1 Guimaraes *et al.* (2001) designed a pair of PCR primers, in accordance with the sequence of a fragment obtained from a chromosomal DNA library clone, by subcloning the portion specifically hybridizing to *C. flaccumfaciens* pv. *flaccumfaciens* in colony and dot-blot experiments. A modification of this protocol has been designed and validated to amplify this target in the target colonies and in bean seed samples, and is described here (López *et al.*, unpublished results).
1.2 Test based on Guimaraes *et al.* (2001).
1.3 The PCR primers were designed in accordance with the sequence of a fragment obtained from a chromosomal DNA library clone, by subcloning the portion specifically hybridizing to *C. flaccumfaciens* pv. *flaccumfaciens* in colony and dot-blot experiments. Analysis of the sequence showed no significant homology with other sequences currently in public databases.
1.4 The following primers were used: forward primer (CF4 5′-CACAGCCACCTACATGC-3′) and reverse primer (CF5

5′-GATCGGGAGTCCGAG-3′) as they were found to produce optimal results.

1.5 Amplicon size 198 bp.

1.6 Enzyme used: 2U Tth DNA polymerase.

1.7 Method developed for a Perkin Elmer 9600 thermocycler.

2. Methods

2.1 Nucleic Acid Extraction and Purification

There is no need to perform DNA extraction on pure cultures. A suspension of the pure culture to be identified is prepared at a concentration of approximately 10^7 cells/mL heated at 95°C for 10–15 min, and 5 µL of the suspension are used as a target DNA.

For seed extracts, the isopropanol method described by Llop *et al.* (1999) is used for extracting DNA before amplification. The same DNA extraction methods described in the protocol of Tegli *et al.* (2002) could also be used. 5 µL of extracted DNA are also used for amplification.

2.2 Polymerase Chain Reaction (PCR) (concentration per single reaction volume)

2.2.1 Total reaction volume of a single PCR reaction 50 µL

5 µL of PCR buffer
1.5 mM $MgCl_2$
0.1 mM dNTPs
2 U Tth DNA polymerase
0.1 µM forward primer
0.1 µM reverse primer
5 µL DNA.

2.2.2 PCR cycling parameters

Initial denaturation 94°C for 4 min, then 40 cycles (93°C for 45 s, 60°C for 45 s, 72°C for 60 s extension, then a final extension at 72°C for 10 min.

2.2.3 Amplification products were separated by agarose gel electrophoresis, stained with ethidium bromide and visualized under UV light. A specific fragment of 198 bp should be amplified.

3. Essential Procedural Information

3.1 Controls:

For a reliable test result to be obtained, the following (external) controls should be included for each series of nucleic acid isolation and amplification of the target organism and target nucleic acid, respectively.

- Negative isolation control (NIC) sterile distilled water and DNA extracted by washings of healthy bean seeds.
- Positive isolation control (PIC) to ensure that nucleic acid of sufficient quantity and quality is isolated: nucleic acid extraction and subsequent amplification of the target organism or a sample that contains the target organism (e.g. naturally infected host tissue or host tissue spiked with the target organism).
- Negative amplification control (NAC) to rule out false positives due to contamination during the preparation of the reaction mix: amplification of PCR grade water that was used to prepare the reaction mix.
- Positive amplification control (PAC) to monitor the efficiency of the amplification: amplification of nucleic acid of the target organism. This can include nucleic acid extracted from the target organism, total nucleic acid extracted from infected host tissue, whole genome amplified DNA or a synthetic control (e.g. cloned PCR product).

3.2 Interpretation of results

A sample will be considered positive if it produces an amplicon of 198 bp, provided that the contamination controls (NIC, NAC) are negative.

A sample will be considered negative if it produces no band, or a band of a different size (not 198 bp), provided that the PIC and the PAC are positive.

Tests should be repeated if any contradictory or unclear results are obtained.

4. Performance criteria available

4.1 Analytical sensitivity data:

Sensitivity was assessed as 100 cfu/mL.

4.2 Analytical specificity data

When tested in PCR reactions using pure DNA from a range of micro-organisms from plants, including bacterial bean pathogens, these primers were shown to be highly specific for *C. flaccumfaciens* pv. *flaccumfaciens*. This test has been developed using 18 strains of *C. flaccumfaciens* pv. *flaccumfaciens* isolated from Germany, Hungary, Romania, Kenya and the United States, and was shown not to cross-react with *C. flaccumfaciens* pv. *betae*, *C. flaccumfaciens* pv. *oortii*, *C. flaccumfaciens* pv. *poinsettiae*, *C. albidium*, *C. citreum*, *C. luteum*, *C. plantarum*, *C. pusillum*, *C. michiganensis* subsp. *insidiosus*, *C. michiganensis* subsp. *michiganensis*, *C. michiganensis* subsp. *nebraskensis*, *C. michiganensis* subsp. *tessellarius*, *Rathayibacter iranicus*, *R. tritici*, *Pectobacterium carotovorum*, *P. cichorii*, *P. marginalis* pv. *marginalis*, *P. syringae* pv. *glycinea*, *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *syringae*, *P. syringae* pv. *tomato*, *X. axonopodis* pv. *phaseoli* and *X. campestris* pv. *vignicola.*

4.3 Data on Repeatability

Not available

4.4 Data on Reproducibility

Not available

Appendix 4 – Pathogenicity tests

Method A

A minimum of 10 bean plantlets of a susceptible variety or cultivar (i.e. Borlotto varieties) are grown until the 2–6 true leaf stage. Ten plantlets are used for each suspected isolate (ideally all isolates, but if this is not possible then a minimum of 5 Gram-positive isolates), in order to achieve a more robust

response and avoid possible misinterpretation of symptoms on single plants. A suspension of the pure culture, suspected to be *C. flaccumfaciens* pv. *flaccumfaciens*, is prepared in sterile water at a concentration approximately 10^8 cfu/mL. A small wound is cut on the stem, just underneath the two cotyledonal leaves, and a droplet of suspension is injected onto the wound. The wound is then sealed with a drop of sterile liquid paraffin. Direct injection is also possible. As a negative control, 10 plants are inoculated in the same way with sterile water, while as positive control 10 further plants are inoculated with a known *C. flaccumfaciens* pv. *flaccumfaciens* virulent strain. Plantlets are then kept in a climatic chamber at a humidity rate of approximately 80%, light photoperiod of 14 h and a temperature of 22–24°C (optimal temperature). The temperature has a major role in development of the disease, and should be maintained within 22–26°C, and in any case should never exceed 28°C. Plantlets should be kept under observation for symptom development for up to 15 days. If, after this period, symptoms are not present, the culture cannot be confirmed as a pathogenic form of *C. flaccumfaciens* pv. *flaccumfaciens*.

Method B

Hsieh *et al.* (2003) showed that the hilum injury/seed inoculation method was simple and effective. At least 10 seeds of each cultivar or line are injured by piercing the hilum with a sterile needle, then soaked in bacterial suspension for 1 h. The inoculated seeds are preferably planted in soil-free substrate, e.g. perlite or Cornell Peat-lite mix (Boodley & Sheldrake, 1977) in Rootrainer™ trays (5 × 14 cells; Spencer-Lemaire Industries, Edmonton, Alberta, Canada), with 1 seed per cell. The inoculated plants are placed in a growth chamber under the conditions described for method A. For negative controls, seeds (at least 10) are injured, soaked in sterile water, and planted in Cornell mix following the method previously described, while for positive controls the same procedure as above is adopted, adding a suspension of virulent *C. flaccumfaciens* pv. *flaccumfaciens* strain in the soaking. Plantlets should be kept under observation for symptom development for up to 3 weeks.

If, after this period, symptoms are not present, the culture cannot be confirmed as a pathogenic form of *C. flaccumfaciens* pv. *flaccumfaciens*.

Method C

A minimum set of 10 bean plantlets of a susceptible variety or cultivar (i.e. Borlotto varieties) per suspected isolate is grown for inoculation. When plantlets have between 2 and 6 true leaves, prepare a suspension of the pure culture of suspected isolates in sterile water at a concentration approximately 10^8 cfu/mL. Inject this suspension with a syringe directly into the stem, making 2–4 injection points spaced out 1 cm apart.

Controls are inoculated in the same way with sterile water, and a series of 10 plantlets is inoculated in the same way with a pure culture of a known strain of *C. flaccumfaciens* pv. *flaccumfaciens*.

Plantlets are then kept in a growth chamber under the conditions described in method A. Plantlets should be kept under observation for symptom development for up to 15 days. If, after this period, symptoms are not present, the culture cannot be confirmed as a pathogenic form of *C. flaccumfaciens* pv. *flaccumfaciens*.